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PARTIAL EXPRESSION OF MONOAMINERGIC (SEROTONINERGIC) PROPERTIES BY THE MULTIPOTENT HYPOTHALAMIC CELL LINE F7. AN EXAMPLE OF LEARNING AT THE CELLULAR LEVEL

F. DE VITRY¹*, J. CATELON¹, M. DUBOIS², J. THIBAUT¹, D. BARRITAU¹,
J. COURTY⁴, S. BOURGOIN⁵ and M. HAMON⁵

¹Neuroendocrinologie Cellulaire et Moléculaire, Collège de France, 11 Place Marcelin Berthelot,
75231 Paris, Cedex 05, France

²INRA, Physiologie de la Reproduction, 37000 Nouzilly, France

³Neurobiologie Cellulaire et Moléculaire, Collège de France, 11 Place Marcelin Berthelot, 75231 Paris,
Cedex 05, France

⁴Biotechnologie, Université Paris Val-de-Marne, 94010 Créteil, France

⁵INSERM U. 288—Neurobiologie Cellulaire et Fonctionnelle, Faculté de Médecine Pitié Salpêtrière,
91 Boulevard de l'Hôpital, 75634 Paris, Cedex 13, France

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Abstract—A serum-free medium supplemented with a glial conditioned medium, a brain extract from 8- to 10-day-old mice, hormones, and eye-derived growth factor has been devised which permitted the mouse primitive hypothalamic nerve cell line F7 to express some biochemical properties typical of monoaminergic neurons. Maximal expression was obtained when the culture conditions were applied for 2 days. Most (90–95%) cells then synthesized [³H]serotonin from [³H]5-hydroxytryptophan (but not from [³H]tryptophan). No synthesis was detected in the presence of carbidopa (20 μ M), therefore suggesting the involvement of L-aromatic-amino-acid decarboxylase in this process. In addition, F7 cells cultured in the involvement of L-aromatic-amino-acid decarboxylase in this process. In addition, F7 cells cultured in such serum-free medium exhibited the capacity of accumulating exogenous serotonin by an ouabain-sensitive mechanism. These data further supported that active molecules in the cell environment can induce, in a primitive cell line, some of the enzymatic activities associated with monoaminergic neurons. Since other well-defined culture conditions can promote the differentiation of the same clone into oligodendrocytes (De Vitry *et al.*, 1983), it can be concluded that the F7 cell has the properties of an embryonic stem cell of the CNS which, depending on external signals, may switch into different alternative developmental neural pathways. We postulate that the stabilization of neuron-like properties due to repetitive cell stimulation by active signals in the environment may represent an example of learning at the cellular level.

INTRODUCTION

Determination of neurochemical specificity is one of the major problems underlying brain development. Although much is known about the features of differentiated neurons, little information has been obtained so far concerning the molecular mechanisms which underlie their emergence during development of the central nervous system (CNS). Cells of the ventricular zone are considered to be the ultimate progenitors of all cell types in the adult CNS (Boulder Committee, 1970). They divide rapidly and after

retiring from their last mitotic cycle, neurons detach and migrate towards their final position.

In the mouse, the hypothalamus begins its development on the 10th day of gestation, soon after the closure of the neural tube, and the majority of hypothalamic neurons cease to divide between embryonic day (ED) 12 and ED 14 (Niimi *et al.*, 1962). Before ED 16, most neurons in the mouse are already grouped into several hypothalamic nuclei (Niimi *et al.*, 1962; Shimada and Nakamura, 1973). Neuronal characteristics, such as the presence of γ -enolase, have been shown to be already present in some ventricular cells before their migration (De Vitry *et al.*, 1980).

*To whom all correspondence should be addressed.

Studies on the early differentiation steps of hypothalamic neurons are hampered by several problems. One is that critical events take place in very small populations of cells; another arises from the fact that major processes of differentiation take place in early stages, that are still largely inaccessible to experimental manipulations *in vivo*. A possible way to get round some of these difficulties is to use stem cell lines as a model system for looking at certain aspects of differentiation *in vitro*. The hypothalamic cell line F7, initially obtained by viral transformation of ED 14 mouse embryos, has been shown to exhibit properties of a primitive nerve cell and potentialities of a pluripotent cell (De Vitry, 1977). We have devised previously a serum-free medium which permits the induction of oligodendroglial differentiation of this primitive cell (De Vitry *et al.*, 1983). Alternatively, after transplantation into mice, F7 cells can switch to neuronal cells synthesizing neurophysin (De Vitry, 1977).

In the present study, we show that under appropriate conditions which may mimic, at least partly, those found during early brain development, the F7 cell line acquires some of the properties typical of serotonergic neurons, such as the capacity of synthesizing serotonin (5-HT) from 5-hydroxytryptophan (5-HTP), and of accumulating exogenous 5-HT by an active uptake process (Osborne, 1982). To our knowledge, this is the first time that a progenitor cell of the CNS has been induced to express—however partially—the properties of differentiated neurons, e.g. those using monoamines as neurotransmitters, under controlled environmental conditions. We postulate that such a commitment (and/or stabilization) of a cellular program represents an example of learning at the cellular level.

EXPERIMENTAL PROCEDURES

Materials

Bovine insulin, human transferrin, progesterone, triiodothyronine (T^3), corticosterone, retinol, N^2, O^6 -dibutyryl cyclic adenosine monophosphate (dibutyrylcyclic AMP), ethanolamine and ouabain were purchased from Sigma (St Louis, Mo., U.S.A.). Synthetic human parathyroid hormone (sequence 1–34) was generously given by Dr Moutard (Hôpital St Antoine, Paris, France).

Other drugs were: *d*-lysergic acid diethylamide (LSD, Sandoz, Basel, Switzerland), pargyline (Abbott, Chicago, U.S.A.), *p*-chlorophenylalanine methylester (pcpa, Labkemi, A.B., Göteborg, Sweden), carbidopa, reserpine (Ciba-Geigy, Basel, Switzerland), fluoxetine (Eli Lilly & Co, Indianapolis, U.S.A.) and citalopram (H. Lundbeck & Co, Copenhagen, Denmark).

The labeled indoles L-tryptophan-[side chain-2,3- $^3H(N)$] (3H)tryptophan, 11.2 Ci/mmol, New England Nuclear, Boston, Mass., U.S.A.), DL-5-hydroxy-[G- 3H]tryptophan

(3H)5-HTP, 5.2–11 Ci/mmol, Amersham International, Amersham, Bucks., U.K.) and (3H)5-hydroxytryptamine (3H)5-HT, generally labeled, 10.2–15.9 Ci/mmol, Amersham International) were purified by ion-exchange chromatography just before use (Hamon *et al.*, 1974; Héry *et al.*, 1979).

Cell cultures

Serum-containing medium (MS^+). Cells were grown in Ham's F10 supplemented with 15% heat-inactivated horse serum (IBF, Paris, France), 2.5% fetal calf serum and 3 mM L-glutamine as previously described (De Vitry, 1977).

Serum-free media (MS^- and MSN). Cells were first seeded at $3-4 \times 10^4$ cells/35 mm tissue-culture dish (Falcon, Becton Primaria) in serum containing medium MS^+ plus F7 conditioned medium (1:1, by vol), and cultured for 24 h at 37°C at saturating humidity in 10% CO_2 -90% air atmosphere. Then the medium was changed to one of the following serum-free media: either the "control" medium (MS^-) (De Vitry *et al.*, 1983) or the "experimental" medium (MSN) consisting of a mixture of Ham's F12, Dulbecco's modified Eagle medium (DME), and C6 Leibovitz modified Eagle medium (1:2:1, by vol) (Table 1). The L15 L15-conditioned medium (1:2:1, by vol) (Table 1). The L15 conditioned medium was obtained by cultivating for 3 days confluent C6 glial cells in bottles (75 cm²) containing 12 ml of L15 and filtering the resulting culture medium through 0.22 μ m Millipore filters. The "experimental" MSN medium was supplemented with various hormones and constituents as described in Table 1. All constituents except insulin, transferrin, L-glutamine and C6-conditioned medium, were added every day in 0.4 ml of Ham's F12 and DME (1:2, by vol), replacing 0.4 ml of cultured medium. Steroid hormones, triiodothyronine and retinol were added in an ethanolic solution (1 μ l/ml).

Preparation of brain extracts

Brains of Swiss mice at various ages (adult, 8–10 days postnatal, neonatal or ED 18) were dissected and homogenized in 5 vol (w/v) of ice-cold saline buffered with 10 mM Na^+ phosphate (pH 7) using a Polytron disrupter. Homogenates were centrifuged at 105,000 g for 30 min at 4°C, and an aliquot (0.1 ml) of supernatants was added to culture dishes each day.

Preparation of eye-derived growth factor (EDGF)

EDGF was prepared from bovine retina, as described elsewhere (Barritault *et al.*, 1982). The fraction used was the "acetic acid purified EDGF" (Barritault *et al.*, 1982). Maximal inductive effect was obtained by adding 15 μ l of EDGF (1000 stimulating units/ml) to each culture dish (total volume: 2 ml).

Immunocytochemical techniques

The antiserum presently used was raised in a rabbit with 5-HT coupled to bovine serum albumin (BSA) by formaldehyde, according to the procedure of Steinbusch *et al.* (1978). The presence of 5-HT antibodies was attested by the positive precipitin ring test and micro-complement fixation (Wasserman and Levine, 1961) using 5-HT coupled to ovalbumin (without cross-reaction with BSA) by formaldehyde as the antigen. Furthermore an ELISA test (Voller *et al.*, 1979) was developed by binding the antiserum (dilution 1/16,000) to polystyrene tubes coated with 5-HT-ovalbumin. Using this test, ovalbumin was totally inactive, 6.4 ng of 5-HT-ovalbumin (corresponding to 1 nM

Table 1. Composition of media

MS^+
Ham's F10:F7-conditioned medium
L-Glutamine, 3 mM
Horse serum, 15%
Fetal calf serum, 2.5%

Cells were first seeded in MS^+ resulted in a reduction of 3H DME = Dulbecco's modified 3H SU = stimulating unit.

5-HT) gave half displacement antigen and 0.5 μ M of 5-HT produce the same half displacement. 5-methyl-tryptamine and active in the micromolar range but tryptophan, 5-HTP, indole acetic acid, tryptamine were totally inactive at description of the species presently used has been (Dubois, 1985).

For the immunocytochemical studies, the indirect method was used with minor modifications on plastic dishes were pargyline and then for 24 h, ouabain (0.5 μ M) as pargyline. Cells were then for 2 h in 6% neutral formalin containing 1 mM $MgSO_4$ (pH 7.4). After a further wash, cells were subsequently exposed for 15–17 h at 4°C (1:400 for the complete inactivation for 30 min with Na^+ phosphate buffer anti-rabbit sheep immunoperoxidase (Institut Pasteur, Paris) activity was then revealed by benzidine as described by Steinbusch (1978). Specificity of the reaction was tested with rabbit serum, as well as adsorbed by an excess of 1 mM).

Incubation with (3H)tryptophan

(3H)Tryptophan (2.5 μ M) (3H)5-HTP (1.5–2.6 μ M) were added to the culture medium (2

An example of learning at the cellular level

Table 1. Composition of media used for culturing F7 cells: serum-containing medium (MS⁺), serum-free medium (MS⁻) and serum-free medium devised for expression of neuronal properties (MSN)

MS ⁺	MS ⁻	MSN
Ham's F10:F7-conditioned medium L-Glutamine, 3 mM Horse serum, 15% Fetal calf serum, 2.5%	Ham's F12:DME† (1:3, by vol) Insulin, 10 µg/ml Transferrin, 25 µg/ml L-Glutamine, 2.4 mM Parathyroid hormone, 2 ng/ml Glucose, 10 mM	Ham's F12:DME†, *C6 L15-conditioned medium (1:2:1, by vol) Insulin, 10 µg/ml Transferrin, 25 µg/ml L-Glutamine, 2.4 mM Parathyroid hormone, 4 ng/ml Ethanolamine, 0.1 mM *D(+)-Galactose, 10 mM *L-Tyrosine, 10 nM *L-Tryptophan, 10 nM *5-HTP, 0.1 µM *Progesterone, 1 nM *Corticosterone, 10 pM *Triiodothyronine, 10 pM *Retinol, 1 nM *Lys-vasopressin, 2.5 IU/ml *Dibutyl cyclic AMP, 100 µM *EDGF, 7.5 SU†/ml *8- to 10-day-old mice brain extract, 50 µl/ml *LSD, 10 µM

Cells were first seeded in MS⁺ and cultured for 24 h. Then the medium was changed to MS⁻ or MSN. Deletion of any single* constituent resulted in a reduction of cell attachment, survival and induction of 5-HT uptake capacity.

†DME = Dulbecco's modified Eagle medium.

‡SU = stimulating unit.

5-HT) gave half displacement of the specifically bound antigen and 0.5 µM of uncoupled 5-HT was necessary to produce the same half displacement. Free tryptamine, 5-methyl-tryptamine and 5-methoxytryptamine were also active in the micromolar range (4 µM < IC₅₀ < 12 µM), but tryptophan, 5-HTP, 5-methoxytryptophan, 5-hydroxyindole acetic acid, tryptophol and 5-hydroxytryptophol were totally inactive at least up to 0.1 mM. A detailed description of the specificity of the 5-HT antiserum presently used has been reported elsewhere (Tillet and Dubois, 1985).

For the immunocytochemical detection of 5-HT in cultured cells, the indirect immunoperoxidase technique was used with minor modifications (De Vitry, 1977). Cells grown on plastic dishes were treated for 30 min with 10 µM pargyline and then for 30 min with 5-HT (20 µM). When mentioned, ouabain (0.5 mM) was added at the same time as pargyline. Cells were then rapidly rinsed and fixed at 4°C for 2 h in 6% neutral formaldehyde or paraformaldehyde containing 1 mM MgSO₄ in 10 mM Na⁺ phosphate buffer (pH 7.4). After a further rinse for 2 h in the same buffer, cells were subsequently exposed to the specific antiserum for 15–17 h at 4°C (1:400 final dilution containing 0.1% BSA for the complete inactivation of BSA antibodies), washed for 30 min with Na⁺ phosphate buffer, and incubated with anti-rabbit sheep immunoglobulins coupled to horseradish peroxidase (Institut Pasteur; 1:80 final dilution). Peroxidase activity was then revealed with H₂O₂ and 3,3'-diaminobenzidine as described previously (De Vitry, 1977). The specificity of the reaction was tested by using preimmune rabbit serum, as well as the specific antiserum previously adsorbed by an excess of 5-HT (at a final concentration of 1 mM).

Incubation with [³H]tryptophan or [³H]5-HTP

[³H]Tryptophan (2.4–3.6 µM final concentration) or [³H]5-HTP (1.5–2.6 µM final concentration) was added to the culture medium (2 ml/dish) immediately after transfer-

ring cells to serum-free conditions, and then added each day thereafter. For these experiments, the corresponding "cold" precursor compounds (see Table 1) were omitted. On the last day of incubation, 0.2 ml of 4 N HClO₄ were added to each culture dish 2 h after supplying the medium with the radioactive precursor. Cell lysate was collected and then pooled with a further wash of the culture dish with 0.8 ml of 0.4 N HClO₄. The mixture was supplemented with 5 mM ascorbic acid and 2 µg 5-HT, and adjusted to pH 6.5 with 0.2 M KH₂PO₄/K₂HPO₄ (pH 5.5) containing 7.5% KOH (w/v). After centrifugation (8000 g, 15 min, at 4°C), each clear supernatant was then passed through a microcolumn (1.3 cm high, 0.2 cm dia) of Dowex AG 50 WX4 buffered at pH 6.5 with 0.2 M Na⁺ phosphate. The subsequent steps were then as described in detail by Héry *et al.* (1979). Briefly, [³H]5-HT adsorbed onto the Dowex column was eluted with 1 ml of a 2 N HClO₄/ethanol solution (1:2, v/v) and the eluate was neutralized with 0.2 M KH₂PO₄/K₂HPO₄ (pH 5.5) containing 7.5% KOH. The KClO₄ precipitate was discarded by centrifugation and [³H]5-HT in the clear supernatant was adsorbed onto an Amberlite (CG50) column (1.5 cm high, 0.4 cm dia). [³H]5-HT was finally eluted with 2 ml of 0.5 M Na⁺ borate buffer (pH 10) and estimated by liquid scintillation counting of an aliquot (1 ml) of the eluate mixed with 10 ml of Aquasol[®] (New England Nuclear).

Under such conditions, the radioactivity finally recovered in "blank" samples (starting with 20–30 µCi pure [³H]tryptophan or [³H]5-HTP) was about 100 cpm, i.e. 0.1 nCi. The spectrophotofluorimetric estimate of "cold" 5-HT added to each sample indicated an average recovery of 60% for the indoleamine (Héry *et al.*, 1979).

Incubations with [³H]5-HT

Cells were maintained under various conditions (MS⁺, MS⁻, MSN) for 2 days before the addition of 1.0 µCi of purified [³H]5-HT (final concentration: 30 nM). The incubation was stopped 30 min later by the addition of 0.2 ml of

Sham International,
[³H]5-hydroxytryptamine
25.9 Ci/mmol, Amer-
by ion-exchange chro-
2 *et al.*, 1974; Héry *et al.*,

Cells were grown in
heat-inactivated horse
fetal calf serum and 3 mM
ed (De Vitry, 1977).

MSN). Cells were first
culture dish (Falcon,
ing medium MS⁺ plus F7
and cultured for 24 h at
10% CO₂-90% air atmos-
changed to one of the follow-
"control" medium (MS⁻)
"experimental" medium
of Ham's F12, Dulbecco's
(ME), and C6 Leibovitz
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6-conditioned medium, were
Ham's F12 and DME (1:2,
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e-cold saline buffered with
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rowth factor (EDGF)

m bovine retina, as described
1982). The fraction used was the
(Barritault *et al.*, 1982). Maxi-
fied by adding 15 µl of EDGF
to each culture dish (total

ed was raised in a rabbit with
rum albumin (BSA) by for-
procedure of Steinbusch *et al.*
antibodies was attested by the
and micro-complement fixation
(1961) using 5-HT coupled to
reaction with BSA) by for-
Furthermore an ELISA test
eoped by binding the antiserum
elystyrene tubes coated with
is test, ovalbumin was totally
albumin (corresponding to 1 nM

4 N HClO₄ to each culture dish, and [³H]5-HT was then extracted as described for the experiments with ³H-labeled precursors. In addition, the effluents and washing of the Dowex microcolumns were further chromatographed onto Sephadex G10 columns in order to detect the possible formation of [³H]5-HIAA: details of the protocol used have been reported elsewhere (Hamon *et al.*, 1973). Blanks consisted of samples incubated under exactly the same conditions except that no cells were seeded in the culture dishes. Such controls indicated that no spontaneous degradation of [³H]5-HT occurred for the 30 min incubation period.

Identification of [³H]5-HT

Amberlite eluates were neutralized with 3 N HCl, and aliquots (0.1 ml) were submitted to HPLC on an Ultrasphere IP column (25 cm long, 0.46 cm dia, 5 μ m) using a mobile phase (flow rate = 1 ml/min) consisting of 70 mM K⁺ phosphate, 2 mM triethylamine, 0.1 mM EDTA and 12% methanol, adjusted to pH 5.1 with citric acid. The elution of 5-HT was monitored by electrochemical detection at 0.65 V. The column eluate was collected as successive 0.5 ml fractions which were mixed with 10 ml Aquasol[®] for radioactivity counting.

Determination of met-enkephalin content

Cells of 20 tissue culture dishes grown for 2 days in the presence (MS⁺) or the absence (MSN) of serum were collected, rinsed twice with 0.1 M Na⁺ phosphate buffer (pH 7.4) containing 0.9% NaCl, and sonicated in 0.1 N HCl. Their met-enkephalin content was then measured using a specific radioimmunoassay (Cesselin *et al.*, 1982). The reliable sensitivity of this assay is 0.1 pg.

Proteins were determined using the Folin phenol procedure (Lowry *et al.*, 1951) with BSA (Sigma) as the standard.

Statistical analysis

Differences were considered as being statistically significant when the Student's *t*-test gave a *P*-value < 0.05.

RESULTS

Morphology of the clonal cell line grown in the presence or absence of serum

F7 cells exhibited a flat morphology when grown in serum-containing medium (MS⁺) (Fig. 1a) (see also De Vitry, 1977).

In MS⁻ medium, cell growth was approximately the same as in MS⁺ medium. However MS⁻ medium did not permit F7 cells to attach to the plastic dish. Seeding in MS⁺ medium was necessary to achieve cell attachment before any experiment could be run in serum-free medium, as already observed for oligodendroglial differentiation of F7 cells in serum-free medium (De Vitry *et al.*, 1983). This led us to culture all cells for 24 h in the MS⁺ medium before selecting other culture conditions (see Experimental Procedures).

In contrast to F7 cells grown in MS⁺ or MS⁻

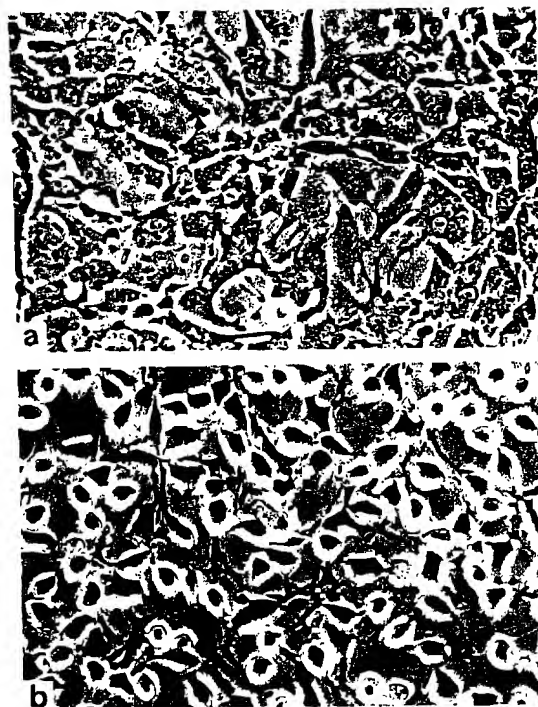


Fig. 1. F7 cells: phase contrast. Cells grown in: (a) MS⁺ medium, (b) MSN medium; $\times 320$.

medium, those grown in the serum-free medium MSN supplemented with hormones, 8- to 10-day-old mice brain extract, and EDGF, became bipolar or multipolar with extended short processes, provided seeding of cells was done at low density (3.4×10^4 cells/dish) (Fig. 1b).

Immunohistochemical evidence of 5-HT uptake capacity in F7 cells cultured in MSN medium

In contrast to F7 cells grown in the presence of serum (MS⁺) or in basal serum-free medium (MS⁻) which gave no positive staining, those maintained for 2 days in MSN medium exhibited a marked immunoperoxidase staining for 5-HT provided they were preincubated for 30 min with 20 μ M of the indoleamine. Staining appeared to be mainly concentrated at one or two poles of the cytoplasm near the surrounding cytoplasmic membrane (Fig. 2a). In contrast, no positive immunocytochemical reaction was noted in F7 cells which kept the flat morphology typical of MS⁺ or MS⁻ culture conditions; usually, such flat cells represented <5% of the total cell population under MSN culture conditions. As expected for a specific staining, all cells remained uncolored when the immunocytochemical reaction was carried out with a preimmune serum or with



Fig. 2. Immunocytochem clonal cell line grown in M for 30 min with 20 μ M : 5-HT antiserum; (b) co previously adsorbed by 5-HT treatment of cell

the 5-HT antiserum γ indoleamine (Fig. 2b).

As illustrated in Fig. observed when ouabai culture medium 30 mi contrast, specific inhibi serotonergic neuron: (Wong *et al.*, 1983) o 1982) did not prevent genous indoleamine in conditions.

Synthesis of [^3H]5-HT from [^3H]5-HTP but not from [^3H]tryptophan in F7 cells cultured in MSN medium

In contrast to cells cultured in the presence of serum (MS⁺), those maintained for 2 days in MSN medium were able to convert [^3H]5-HTP into [^3H]5-HT (Table 2). Identification of [^3H]5-HT as the final product was made by ion-exchange chromatography and HPLC analysis, as shown in Fig. 3. Further confirmation of the synthesis of the indoleamine [^3H]5-HT was obtained by the addition of 20 μM carbidopa into the culture medium since the resulting blockade of L-aromatic-amino-acid decarboxylase (AADC) activity was associated with the loss of cell capacity to decarboxylate [^3H]5-HTP (Table 2). In contrast, monoamine oxidase inhibition by 10 μM pargyline did not affect the [^3H]5-HT content in culture dishes supplemented with [^3H]5-HTP for 3 days (Table 2, see Experimental Procedures). The lack of monoamine oxidase activity in F7 cells was confirmed using exogenous [^3H]5-HT: no significant conversion of [^3H]5-HT into [^3H]5-hydroxyindole acetic acid ([^3H]5-HIAA) occurred for a 30 min incubation of F7 cells with the [^3H]indoleamine. Similar negative findings were ob-

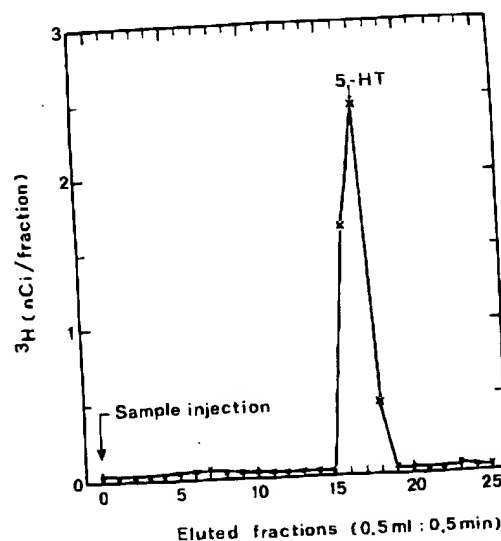


Fig. 3. HPLC elution profile of [^3H]5-HT synthesized from [^3H]5-HTP by F7 cells grown in MSN medium. Cells were grown for 2 days in MSN medium (2 ml) supplemented with 13 μCi [^3H]5-HTP, and newly synthesized [^3H]5-HT was extracted as described in Experimental Procedures. The HPLC elution profile of [^3H]5-HT was established by counting the radioactivity of each collected fraction (0.5 ml). The elution volume of authentic 5-HT (8.5 ml) was monitored by electrochemical detection ($E = 0.65\text{ V}$). Similar results were obtained in 5 independent experiments.

Fig. 2. Immunocytochemical detection of 5-HT in the F7 clonal cell line grown in MSN medium. Cells were incubated for 30 min with 20 μM 5-HT before fixation ($\times 480$). (a) 5-HT antiserum; (b) control with 5-HT antiserum previously adsorbed by 5-HT; (c) 5-HT antiserum after 1 h treatment of cells with 0.5 mM ouabain.

the 5-HT antiserum previously adsorbed with the indoleamine (Fig. 2b).

As illustrated in Fig. 2c, uncolored cells were also observed when ouabain (0.5 mM) was added to the culture medium 30 min before 5-HT (20 μM). In contrast, specific inhibitors of 5-HT uptake in central serotonergic neurons such as 10 μM fluoxetine (Wong *et al.*, 1983) or 10 μM citalopram (Hyttel, 1982) did not prevent the accumulation of the exogenous indoleamine in F7 cells under MSN culture conditions.

Table 2. Differential use of [3 H]5-HTP, [3 H]tryptophan and [3 H]5-HT by F7 cells cultured under various conditions

Added substrate	Culture conditions	[3 H]5-HT (nCi)
[3 H]5-HTP (16 μ Ci)	MS ⁺	ND
	MSN	51.61 \pm 4.02
	MSN + carbidopa (20 μ M)	0.46 \pm 0.09
	MSN + pargyline (10 μ M)	58.86 \pm 5.31
[3 H]tryptophan (20 μ Ci)	MS ⁺	ND
	MSN	ND
[3 H]5-HT (1.0 μ Ci)	MS ⁺	936 \pm 60
	MSN	948 \pm 72

In all cases, F7 cells were grown for 24 h in the presence of serum (MS⁺) to allow their attachment to the culture dishes, and then for 48 h either in the same medium (MS⁺) or in the serum-free medium supplemented with inducing factors (MSN). [3 H]5-HTP or [3 H]tryptophan was added each day up to a total content of 16–20 μ Ci/culture dish (2 ml of medium). [3 H]5-HT was added 30 min before the addition of 0.2 ml of 4 N HClO₄ to stop the reaction (see Experimental Procedures). The total content of [3 H]5-HT was measured in each case and expressed as nCi/dish. Each value is the mean \pm SEM of 6 separate determinations. ND = not detectable.

tained with cells cultured in the presence (MS⁺) or absence (MS⁻, MSN) of serum.

As shown in Table 2, no synthesis of [3 H]5-HT was found when [3 H]tryptophan was used as the labeled substrate in any of the culture conditions presently studied (MS⁺ or MSN medium).

Effects of reserpine treatment or K⁺-induced depolarization on the accumulation of [3 H]5-HT newly synthesized from [3 H]5-HTP in cultured F7 cells

After a 2-day culture in MSN medium supplemented with [3 H]5-HTP, newly synthesized [3 H]5-HT was found principally in the culture medium. Indeed only 1.5% of total [3 H]5-HT was recovered in the cell pellet (Fig. 4). The addition of 1 μ M reserpine to the culture medium 1 h before collecting cells by centrifugation did not alter their [3 H]5-HT content. In contrast, depolarization by an excess of K⁺ (40 mM KCl added 1 h before the centrifugation) resulted in a significant decrease (–36%) of the cellular accumulation of newly synthesized [3 H]5-HT (Fig. 4).

Respective involvement of various MSN constituents for the induction of 5-HT uptake and synthesizing capacities in F7 cells

The effect of hormones and factors on both culture survival and 5-HT uptake and synthesizing capacities was investigated with serum-free medium in which the concentrations of one or two hormones or growth factors were systematically altered compared to the complete MSN medium.

Deletion of any single constituent resulted in a reduction of 5-HT uptake in F7 cells. This is illustrated in Fig. 5 which shows a marked decrease in the immunostaining of F7 cells preincubated with 5-HT

when LSD was omitted from the cultured medium. Similar pictures were obtained when any compound labeled with an asterisk in Table 1 was omitted.

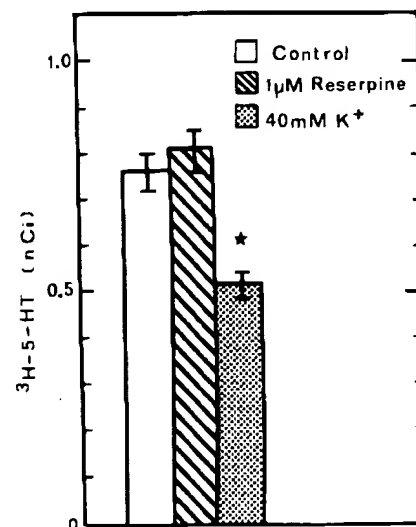


Fig. 4. Effect of reserpine treatment or K⁺-induced depolarization on the [3 H]5-HT cellular content of F7 cells grown in MSN medium supplemented with [3 H]5-HTP. F7 cells were grown for 2 days in MSN medium (2 ml) containing 18 μ Ci of [3 H]5-HTP and then exposed for 1 h to 1 μ M reserpine or 40 mM K⁺. After collection from the culture dishes, cells were centrifuged and the corresponding pellets were washed once with Na⁺ phosphate buffer. Finally [3 H]5-HT was extracted with 0.4 N HClO₄ and estimated as described in Experimental Procedures. Each bar is the mean \pm SEM of 4 independent determinations of [3 H]5-HT (in nCi) found in the cell pellets. The total content of [3 H]5-HT newly synthesized from [3 H]5-HTP was not significantly affected by either treatment (\bar{X} = 44.5 \pm 3.8 nCi, n = 12). * P < 0.05 when compared to respective controls.

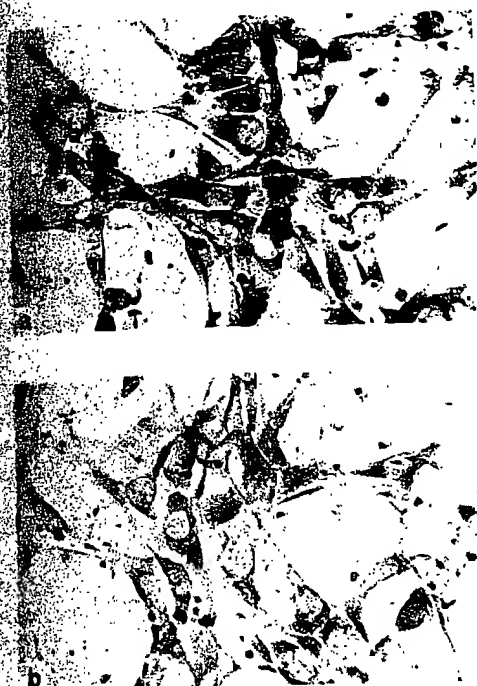


Fig. 5. Effect of LSD deletion from the MSN culture medium on 5-HT uptake capacity of F7 cells ($\times 480$). Cells were preincubated for 30 min with $20 \mu\text{M}$ 5-HT in MSN medium before fixation and immunostaining with the 5-HT antiserum: (a) MSN, (b) MSN without LSD. Note a marked decrease in the immunostaining of F7 cells in the absence of LSD.

Studies of a wide range of concentrations of each compound led to the MSN medium (see the composition in "Table 1") allowing optimal cell attachment and survival, and induction of 5-HT uptake capacity.

One critical factor was the age of the mice from which brain extracts were prepared. Thus, cultures with extracts from ED 18, newborn, 5-day-old, 8- to 10-day-old or adult (~ 90 -day-old) mice revealed that the induction of 5-HT uptake capacity was maximal with extracts from 8- to 10-day-old animals.

Similar experiments conducted to identify which factor(s) was (were) responsible for the induction of $[\text{H}]5\text{-HT}$ synthesizing capacity indicated that complex interactions of several factors probably played much more important roles than any individual factor. A typical example of such an interaction is illustrated in Fig. 6. Although the removal of LSD or EDGF alone exerted only a discrete influence on the capacity of F7 cells to synthesize $[\text{H}]5\text{-HT}$ from $[\text{H}]5\text{-HTP}$, the simultaneous removal of both com-

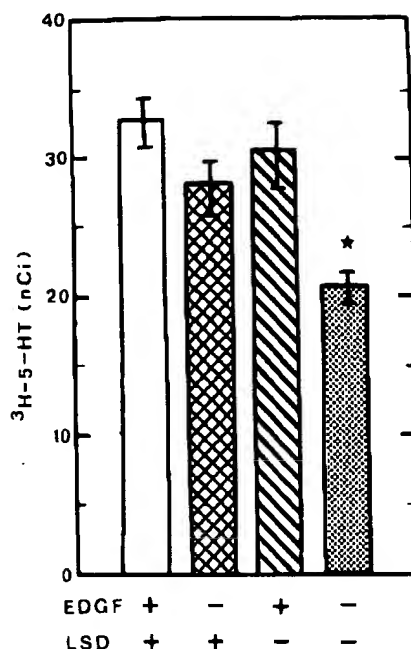


Fig. 6. Effects of EDGF and/or LSD deletion from the MSN culture medium on the synthesis of $[\text{H}]5\text{-HT}$ from $[\text{H}]5\text{-HTP}$ by F7 cells. F7 cells were grown for 2 days either in complete MSN medium or in the same medium deprived of EDGF ($15 \mu\text{l}/2 \text{ ml}$) and/or LSD ($10 \mu\text{M}$) as indicated on the abscissa. The same amount of $[\text{H}]5\text{-HTP}$ ($10 \mu\text{Ci}$) was added to all culture dishes and newly synthesized $[\text{H}]5\text{-HT}$ was then estimated as described in Experimental Procedures. Each bar is the mean \pm SEM of 4 independent determinations. * $P < 0.05$ when compared to $[\text{H}]5\text{-HT}$ synthesis in complete MSN medium (empty bar).

pounds resulted in a marked decrease in this capacity (Fig. 6).

Time-dependent irreversible commitment of F7 cells to take up 5-HT under MSN culture conditions

To determine whether the appearance of 5-HT uptake capacity represented an irreversible commitment towards a monoaminergic lineage or not, F7 cells were first grown for 1 or 2 days in MSN medium and then switched to serum-supplemented culture conditions (MS^+).

Most F7 cells grown in MSN medium for 2 days were then unable to survive in MS^+ medium. Only the flat cells representing 3–5% of the total cell population remained attached to the dish and went on dividing. However, cells that were grown for only 1 day in MSN medium remained attached and survived after the switch to MS^+ medium, but they lost their capacity to accumulate exogenous 5-HT.

Table 3. Met-enkephalin-like material (MELM) in F7 cells cultured in the presence (MS⁺) or absence (MSN) of serum

Culture conditions	MELM (pg/mg protein)
MS ⁺	5.04 ± 1.15
MSN	3.86 ± 0.53

F7 cells were grown for 24 h in MS⁺ medium and then for 48 h either in the same medium or in serum-free medium supplemented with inducing factors (MSN). MELM content of F7 cells was then estimated by radioimmunoassay (Cesselin *et al.*, 1982) and expressed as pg met-enkephalin equivalents/mg protein. Each value is the mean ± SEM of 3–4 independent determinations.

Met-enkephalin content in F7 cells cultured in the presence or absence of serum

In agreement with previous data (Cesselin *et al.*, 1982), we detected met-enkephalin-like immunoreactivity in F7 cells cultured in MS⁺ medium (Table 3). In contrast to 5-HT uptake and synthesizing capacities, the levels of met-enkephalin-like material in F7 cells did not depend upon the culture conditions: similar levels were found in F7 cells grown in MS⁺ or MSN medium (Table 3).

DISCUSSION

The purpose of this investigation was to study the possible commitment of a pluripotent nerve cell line towards a neuronal lineage, and shed some light on the environmental signals involved in this determination. The hypothalamic cell line F7 (De Vitry, 1977) was used here as a model for such pluripotent nerve stem cells in the CNS.

A preliminary step consisting of defining a serum-free medium able to support proliferation of F7 cells for short duration experiments has been previously achieved (De Vitry *et al.*, 1983). On account of the report by Bottenstein and Sato (1979) that neuronal cell lines could survive *in vitro* in the presence of specific combinations of hormones instead of serum, and our own results showing that oligodendroglial differentiated properties could be induced in F7 cells by appropriate serum-free culture conditions (De Vitry *et al.*, 1983), we then decided to pursue our investigations with the development of a serum-free medium which would promote the induction of neuronal properties in primitive nerve cells.

In contrast to F7 cells cultured in the presence of serum, those maintained in this specially devised medium MSN (Table 1) were able to accumulate exogenous 5-HT, and synthesize [³H]5-HT from [³H]5-HTP but not from [³H]tryptophan, showing the presence of AADC (EC 4.1.1.28) but not of tryptophan hydroxylase. By immunocytochemistry, F7

cells with newly acquired 5-HT uptake capacity were readily identifiable in culture 2 days after their transfer into serum-free medium (MSN), provided they were seeded at low density. Instead of the typical morphology observed in the presence of serum, F7 cells cultured in MSN medium exhibited a bipolar or multipolar shape with several extended processes which gave them neuron-like patterns.

In spite of these newly acquired properties, F7 cells could not be considered as serotonergic neurons since they lacked the specific enzyme tryptophan hydroxylase. Furthermore, the uptake process responsible for 5-HT accumulation in F7 cells cultured in MSN medium was inhibited by the Na⁺ pump blocker ouabain, as expected from a functional coupling between Na⁺/K⁺-dependent ATPase and the 5-HT carrier, like in serotonergic neurons (Bogdanski *et al.*, 1970), but not by typical 5-HT uptake blockers such as fluoxetine and citalopram (Wong *et al.*, 1983; Hyttel, 1982).

Other monoaminergic neurons such as dopaminergic, noradrenergic and adrenergic neurons also contain the enzyme AADC and exhibit some ability to take up 5-HT (Shaskan and Snyder, 1970). Accordingly it could be proposed that F7 cells cultured in MSN medium were in fact more catecholaminergic than serotonergic. However immunostaining with anti-tyrosine hydroxylase antiserum (Thibault *et al.*, 1981) was negative, and no ³H-labeled catecholamine was found after a 3-day culture in MSN medium supplemented with [³H]tyrosine. In contrast, these cells did exhibit the capacity to convert [¹⁴C]dihydroxyphenylalanine ([¹⁴C]DOPA) into [¹⁴C]dopamine (unpublished observations), as expected from their expression of AADC.

In addition to well-characterized monoaminergic neurons, other cells have been shown to contain AADC but apparently neither tryptophan hydroxylase nor tyrosine hydroxylase in the CNS (Jaeger *et al.*, 1983, 1984). These cells may correspond at least partly to the APUD cells characterized by Pearse (1969) since they decarboxylate the Amine Precursor (5-HTP or DOPA) which has been taken up. Whether F7 cells cultured in MSN medium belong to the APUD lineage remains an interesting possibility since, like APUD cells, they not only decarboxylate 5-HTP or DOPA but also contained a neuropeptide, namely met-enkephalin (Cesselin *et al.*, 1982; Table of the present paper). Furthermore, Teitelman *et al.* (1983) noted that cells expressing AADC appear very early in the notochord and the neural tube of developing embryo, well before catecholaminergic neurons, and therefore AADC-containing F7 cells

presently studied, which derive from the mouse *en-*... might well belong to this cell type. Alternatively, they might correspond to some precursors of monoaminergic neurons since it has been proposed that AADC is expressed before tyrosine hydroxylase (or tryptophan hydroxylase) in such neurons (Mandelman *et al.*, 1983). More studies are needed to determine if F7 cells really express a preestablished genetic program, due for instance to their hypothalamic origin, or whether the lack of interactions with other environmental cues is responsible for the absence of tryptophan hydroxylase or tyrosine hydroxylase.

The possible relationship between the acquisition by F7 cells of AADC activity and 5-HT uptake capacity, and F7 cell induction to a neuronal or APUD lineage could be further assessed by examining appropriate neuronal markers such as pyruvate kinase and the capacity to bind tetanus toxin. However, these two "specific" markers have been found not only in neurons or APUD cells (Schmechel *et al.*, 1978; Schmechel, 1985), but also in some types of glial cells (Raff *et al.*, 1983; Schmechel, 1985). The presence of the "specific" neurofilament triplet proteins in F7 cells cultured in MSN medium is currently under investigation. However, even with these marker proteins, negative findings would not allow negation of F7 cell commitment to a neuronal lineage since typical neurons without noticeable neurofilaments have been already described (Sharp *et al.*, 1982).

The hormones presently chosen for cultivating F7 cells in the absence of serum have already been shown to play major roles for neural development *in vivo* or *in vitro*, e.g. triiodothyronine, corticosterone, progesterone, vasopressin [see Legrand (1983) for a review, and McEwen (1981), Toran-Alleran (1981) and Auerbach and Lipton (1982)]. Other factors included in MSN medium have been selected on the basis of their reported efficacy to promote the differentiation of various cell types; for instance recent investigations have shown that vitamin A and its derivatives (retinoids) induce the *in vitro* differentiation of teratocarcinoma stem cells (Strickland and Mahdavi, 1978; Solter *et al.*, 1979; Jones-Milleneuve *et al.*, 1982). Similarly, dibutyryl cyclic AMP stimulates various differentiation processes in neurons and glial cells (Waymire *et al.*, 1978; Walicke and Patterson, 1981). In addition, the C6 glioma L15-conditioned medium also participated in the induction of AADC and 5-HT uptake capacity in F7 cells cultivated in MSN medium. Previous reports have already mentioned that C6 glioma cells release

into the culture medium substances which can support the survival (and process formation) of dissociated neurons from the peripheral nervous system (Barde *et al.*, 1978; Unsicker *et al.*, 1984). A brain extract was another necessary requirement for the induction of 5-HT uptake and synthesizing capacities in F7 cells. While an adult brain extract was essential for oligodendroglial differentiation of F7 cells (De Vitry *et al.*, 1983), only the brain extract from 8- to 10-day-old mice was found in the present study to sustain the expression of 5-HT uptake capacity and AADC activity in F7 cells. Fractionation of these extracts should allow the identification of which factors in the brain of adult or 8- to 10-day-old mice were responsible for the induction of oligodendrocyte or monoaminergic characters in F7 cells, respectively.

It is striking that EDGF, a growth factor activity derived from retina (Barritault *et al.*, 1982), is a necessary—although not sufficient—factor for the induction of both oligodendrocyte (De Vitry *et al.*, 1983) and monoaminergic (this paper) properties of F7 cells. Like NGF (Greene and Tischler, 1976; Schubert *et al.*, 1978), EDGF could act by inducing mitotic arrest and activating a differentiation program; alternatively, it might modify the accessibility of membrane receptors to some external signals. Undoubtedly, EDGF acted upon F7 cells in a different way than on other cell types, since it is generally considered as a factor stimulating cell proliferation but not differentiation (Barritault *et al.*, 1982). Recent developments in the purification of EDGF indicate that it is in fact composed of three biologically active growth factors, two of which are now being purified to homogeneity (Courty *et al.*, 1985). Further investigations will be needed to identify which of these molecules was (were) responsible for the effects observed here.

It has been suggested that monoamines can play a neurotrophic role in early ontogenesis, probably via the stimulation of specific receptors on target cells (Hamon *et al.*, 1985). Some support for this concept is found in the present study since we observed that a 5-HT agonist, LSD [see Hamon (1984) for a review], at least in combination with EDGF, was required for the optimal induction of AADC in F7 cells grown in MSN medium. However, further studies with other 5-HT agonists and antagonists are necessary before the possible involvement of specific 5-HT receptors (on F7 cells) can be proposed, on account of the LSD effect.

In conclusion, the present study revealed that under appropriate culture conditions (MSN), a primitive nerve cell line, F7, previously shown to

differentiate into *oligodendrocytes* under other conditions (De Vitry *et al.*, 1983), could express some of the characteristics typical of monoaminergic neurons, e.g. 5-HT uptake capacity and AADC activity. These data provided some support for the possible existence at the early steps of CNS formation of a transitory bipotential cell type which can give rise either to a glial cell (containing specific markers such as carbonic anhydrase II and myelin basic protein) or a neuron- or APUD-like cell in response to environmental cues. Since the different factors in MSN medium had to act synergistically and *repetitively* to induce 5-HT uptake and synthesizing capacity in F7 cells, we can postulate that active signals from the cell environment may trigger a preestablished genetic program and stabilize neuronal functions. We define such mechanisms of *reinforcement* and *stabilization* of the commitment of one cell as a process of *learning at the cellular level*. This *learning* could be of general significance for stabilization of nerve cell identity during brain development.

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